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In re Application of: **Michael Daly *et al.***

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For: **Engineered Radiation Resistant Bioremediating Bacteria**

DECLARATION UNDER 37 C.F.R. 1.131

I, Michael J. Daly declare as follows:

1. I am a named co-inventor of the above-referenced U.S. Patent Application (10/089,175) along with Lawrence P. Wackett.

2. The journal publication entitled "Engineering a recombinant *Deinococcus radiodurans* for organopollutant degradation in radioactive mixed waste environments" published in Nature Biotechnology, volume 16, pages 929 to 933 (copy attached as Exhibit A) reflects work of the above identified co-inventors disclosed in this application.

3. The subject matter of pending claims 1, 2, 12, 25 to 28 and 42 to 51 in this application is evidenced in the journal publication entitled "Engineering a recombinant *Deinococcus radiodurans* for organopollutant degradation in radioactive mixed waste environments" (Exhibit A). This subject matter was invented in the United States before October 1, 1998 but less than one year prior to the earliest priority filing date of September 27, 1999. The subject matter was therefore invented prior to the date of disclosure of the subject matter in the journal publication as evidenced by the date of publication (earliest possible disclosure of October 1, 1998).

4. I further declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were

made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.

Respectfully submitted,

Michael J. Daly

NOV 23, 2005
Date

Engineering a recombinant *Deinococcus radiodurans* for organopollutant degradation in radioactive mixed waste environments

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Thousands of waste sites around the world contain mixtures of toxic chlorinated solvents, hydrocarbon solvents, and radionuclides. Because of the inherent danger and expense of cleaning up such wastes by physicochemical methods, other methods are being pursued for cleanup of those sites. One alternative is to engineer radiation-resistant microbes that degrade or transform such wastes to less hazardous mixtures. We describe the construction and characterization of recombinant *Deinococcus radiodurans*, the most radiation-resistant organism known, expressing toluene dioxygenase (TDO). Cloning of the *tod* genes (which encode the multicomponent TDO) into the chromosome of this bacterium imparted to the strain the ability to oxidize toluene, chlorobenzene, 3,4-dichloro-1-butene, and indole. The recombinant strain was capable of growth and functional synthesis of TDO in the highly irradiating environment (60 Gy/h) of a ^{137}Cs irradiator, where 5×10^6 cells/ml degraded 125 nmol/ml of chlorobenzene in 150 min. *D. radiodurans* strains were also tolerant to the solvent effects of toluene and trichloroethylene at levels exceeding those of many radioactive waste sites. These data support the prospective use of engineered *D. radiodurans* for bioremediation of mixed wastes containing both radionuclides and organic solvents.

Keywords: toluene dioxygenase, bioremediation

In 1990 there were 26 countries that produced electricity from a total of 426 operating nuclear power plants[1], and many countries maintain or are developing nuclear weapons programs. As a result of the last 5 decades of nuclear proliferation, thousands of radioactive waste sites were generated with little regard for public safety or environmental concern[2]. These nuclear production wastes are highly toxic, recalcitrant, and require the development of new technologies for their cleanup.

A detailed 1992 survey of 91 (out of 3000) US Department of Energy (DOE; Germantown, MD) waste sites indicates that about 32% (960) of soils and 45% (1350) of groundwaters at these sites were contaminated with organopollutants (e.g., toluene and trichloroethylene [TCE]) plus radionuclides and heavy metals (e.g., uranium, plutonium, cesium, lead, chromium, and arsenic)[3]. There is currently little prospect for cleanup of these wastes by physicochemical means alone because of the extreme expense, danger, and intensity of labor. Unless new cost-effective cleanup technologies are developed, these wastes increasingly will threaten human health as they leach into the environment. In the United States alone, of the 3000 waste sites disclosed by the DOE, the total cleanup by physicochemical methods was estimated in 1988 at about $90 billion[4] and more recently between $189 and $265 billion, over a 70 year period[5]. Such sites, therefore, represent defined targets for less expensive in situ bioremediation technologies using engineereed microorganisms that can degrade the organic component of radioactive mixed wastes.

Numerous microorganisms (particularly *Pseudomonas* spp.) have been described that have the ability to degrade, transform, detoxify, or immobilize a plethora of organic and inorganic pollutants[6–12]. Biodegradation of the organic component of these sites is a logical first step in their detoxification. Most microorganisms are sensitive to the damaging effects of radiation found in mixtures containing radionuclides. For example, *Pseudomonas* spp. are very sensitive to radiation (more sensitive than *Escherichia coli*[13]) and are not suited to remediate mixed wastes. Therefore, radiation-resistant microorganisms that can degrade organic toxins need to be found in nature or engineered in the laboratory to address this problem.

The most radiation-resistant organism discovered to date is *Deinococcus radiodurans*[14–16]. *D. radiodurans* is a nonpathogenic, desiccation-resistant[17], soil bacterium that can survive acute exposures to ionizing radiation of 15,000 Gy without lethality[18]. This dose induces about 130 double-strand breaks (DSBs) per *D. radiodurans* chromosome[18]. This ability is extraordinary as most organisms cannot survive more than two to three DSBs per chromosome[19]. Previous studies of *D. radiodurans*' resistance to radiation describe its exposure to acute doses of radiation (i.e., delivered as a single dose) followed by its recovery in the absence of radiation[18,20,21]. We show that *D. radiodurans* can express foreign genes while growing in the presence of continuous irradiation. This characteristic is critical given the continuous exposure to radiation and other DNA-damaging agents a microorganism would be subjected to at radioactive DOE waste sites. Recent advances in our ability to genetically manipulate this bacterium[22] have led to insights into its DNA repair capabilities. Its radiation resistance has been shown to be, in part, attributable to exceedingly efficient *recA*-depen-

dent[18,20,23,24] as well as *recA*-independent DNA repair processes[21]. Likewise, *D. radiodurans* is also extraordinarily resistant to most chemical DNA damaging agents such as nitrous acid, 4-nitroquinoline-*N*-oxide, and mitomycin-C[15,25,26].

Following exposure to any amounts of these DNA-damaging agents, including highly lethal exposures, *D. radiodurans* survivors show approximately the same low level of mutation frequency that ordinarily occurs during a normal round of replication (a spontaneous mutation frequency of 10^{-3}–10^{-9} per gene per generation, depending on the particular gene)[25]. Any DNA introduced artificially into *D. radiodurans* as plasmids or chromosomal insertions are equally protected from mutations[18,20,21,24], making it an ideal candidate for expression of bioremediating proteins in genotoxic environments. The sequencing of the *D. radiodurans* genome at The Institute for Genomic Research[27] increases the attractiveness of this organism for genetic engineering. Currently, there are no reports of organopollutant-degrading genes, either native or recombinant, being expressed in any radiation-resistant organism.

A number of the bacterial enzymes that initiate the attack on organopollutants are oxygenases requiring metal and/or organic cofactors, thus posing many potential problems with expression in exotic host bacteria. To determine if *D. radiodurans* can functionally synthesize cloned multicomponent enzymes for biodegradation purposes, we tested *D. radiodurans* as a host for expression of toluene dioxygenase (TDO) from *Pseudomonas putida* F1[12]. A functional TDO requires the coordinate expression of four genes (*todC1C2BA*) and the assembly of three protein components: a flavoprotein, a ferredoxin containing a Rieske-type [2Fe-2S] center, and a terminal oxygenase containing an iron-sulfur center and a nonheme ferrous iron center[28]. We describe the construction of a *D. radiodurans* strain containing the *tod* genes, and characterization of the functional expression of TDO by this recombinant strain in both nonradioactive and highly radioactive environments.

Results

Sequence analysis of the *D. radiodurans* genome. The nearly completed *D. radiodurans* genomic DNA sequence[29] was searched for similarity to TDO sequences using the Basic Local Alignment Search Tool (BLAST). No *D. radiodurans* sequences were found to have high levels of homology at the DNA or at the peptide level, suggesting that a TDO homolog does not exist in *D. radiodurans* strain R1 (wild type).

Growth of *D. radiodurans* (wild type) in the presence of radiation. Over a period of 30 h, *D. radiodurans*' growth characteristics and viability were not affected by the continual presence of 60 Gy/h radiation in a ^{137}Cs irradiator (Fig. 1); this level of continuous radiation exceeds those commonly found at waste sites[3]. *D. radiodurans* strains reached the stationary phase of their growth irrespective of the presence or absence of γ-irradiation. By comparison, *E. coli* (wild type: AB1157) did not grow and was killed by this level of radiation exposure, as expected.

Construction of *D. radiodurans* strains MD560 (*tod*$^+$) and MD417 (*tod*$^-$). An EcoRI-BamHI (4.2 kb) fragment containing the *todC1C2BA* genes[12] was cloned from plasmid pHG2[11] into the *D. radiodurans* chromosomal tandem duplication vector pMD417[21] (Fig. 2) forming plasmid pMD532. pMD417 contains a single EcoRI and BamHI site in the *tet* gene. By cloning *todC1C2BA* into the EcoRI-BamHI sites of pMD417, the *tod* genes were placed under the control of a constitutive *D. radiodurans* promoter (Fig. 2). pMD532 was transformed into *D. radiodurans* R1 followed by selection on tryptone, glucose, yeast extract (TGY) plates[18] containing kanamycin. Strain MD560 was selected and the restriction endonuclease map of its chromosomal integration site (Fig. 2) was confirmed by Southern blot analysis (data not shown). The *tod* genes were present at about two copies per chromosome (8–20 copies per cell; *D. radiodurans* has 4–10 identical chromosomal copies per cell[25])(data not shown).

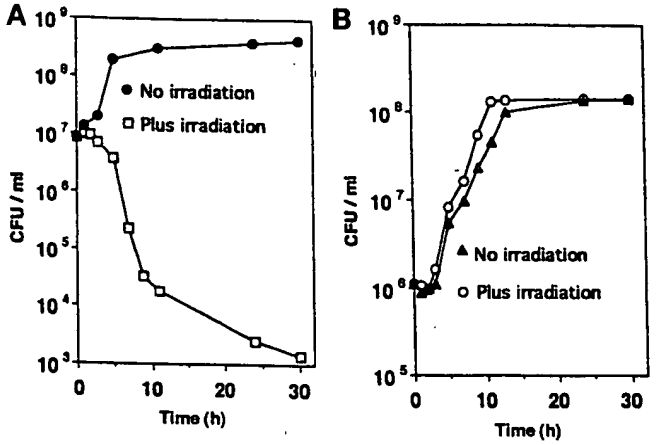

Figure 1. Effect of γ-irradiation (60 Gy/h) on the growth of (A) *E. coli* and (B) *D. radiodurans* R1 expressed as colony forming units (cfu).

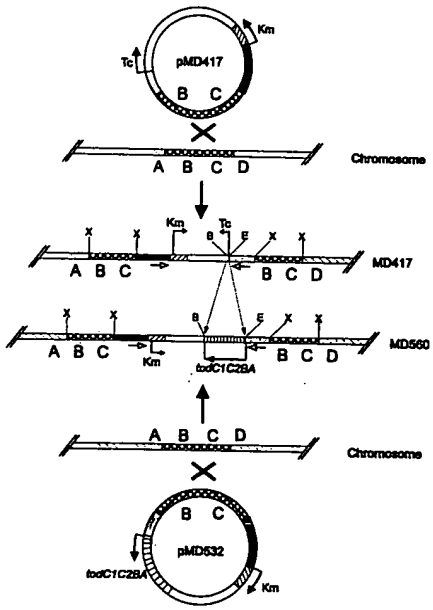

Figure 2. Chromosomal maps of *D. radiodurans* strains MD417 (*tod*$^-$) and MD560 (*tod*$^+$). MD560 constitutively expresses TDO (encoded by *todC1C2BA*). MD417 is a control strain lacking *tod* genes. The strains are wild-type strain R1 transformed with the circular plasmids pMD532 and pMD417, respectively. The two arrows between chromosomal regions MD417 and MD560 show the location of the *tod* genes; the black arrow below the *tod* genes shows the transcriptional direction. bc: the chromosomal integration sequence; a and d: chromosomal sequences flanking the integration site bc. Km: resistance to kanamycin encoded by the *aphA* gene (diagonally hatched segment). *aphA* gene transcription is driven by a deinococcal constitutive promoting sequence (open arrow) located in the black segments. Tc: resistance to tetracycline is encoded by the *tet* gene (white region). Transcription of the *tet* and *todC1C2BA* genes is driven by a deinococcal constitutive promoting sequence (open arrow) present in the light grey segments. Restriction enzyme sites are X: XbaI; B: BamHI; and E: EcoRI.

Product identification. The *todC1C2BA* genes cloned into *D. radiodurans* (strain MD560) are constitutively expressed to make functional TDO. *D. radiodurans* strains R1 (wild type), MD417, and MD560 were incubated with indole[30]; only strain MD560 yielded indigo. For detailed analysis of products, incubations were carried out overnight with mid-log phase grown *D. radiodurans* strains MD560

Table 1. Mass spectra and GC retention time (R_t) of products identified from incubations of *D. radiodurans* MD560 (*tod*$^+$) or purified toluene dioxygenase with the listed substrates.

Starting compound	Transforming organism or enzyme	Product data: GC R_t (minutes)	Product data: *m/z* (% relative intensity)
toluene *cis*-dihydrodiol	none	9.22	126 (10), 108 (100), 107 (49), 80 (67), 79 (84)
toluene	*D. radiodurans* MD560	9.25	126 (10), 108 (100), 107 (50), 80 (65), 79 (84)
chlorobenzene	toluene dioxygenase	11.15	148 (10), 146 (30), 130 (25), 128 (71), 117 (19), 102 (39), 100 (100)
chlorobenzene	*D. radiodurans* MD560	11.14	148 (8), 146 (27), 130 (19), 128 (57), 117 (19), 102 (39), 100 (100)
3,4-dichloro-1-butene	toluene dioxygenase	NA	163 (3), 161 (16), 159 (19), 145 (14), 143 (61), 141 (100)
3,4-dichloro-1-butene	*D. radiodurans* MD560	NA	163 (2), 161 (11), 159 (20), 145 (9), 143 (66), 141 (100)

NA: not applicable, compounds analyzed by direct probe insertion.

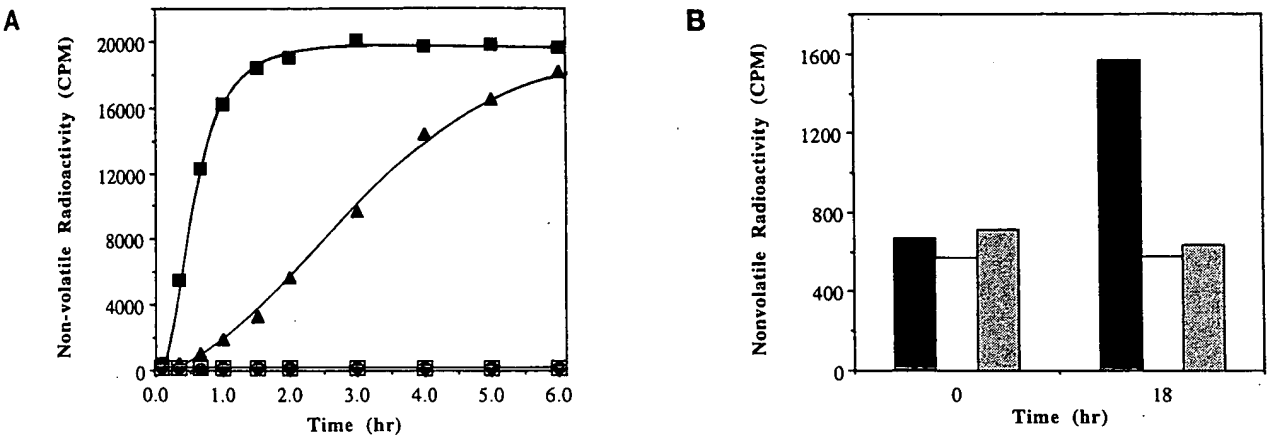

Figure 3. Nonvolatile radioactive products formed. (A) Upon incubation of ^{14}C-labeled toluene with *D. radiodurans* strains MD560 (*tod*$^+$; ▲) and MD417 (*tod*$^-$; □), *E. coli* (pDTG351; *tod*$^+$; ■), and negative control of TGY medium alone (●). (B) Upon incubation of ^{14}C-labeled trichloroethylene with *D. radiodurans* strains MD560 (*tod*$^+$; black), MD417 (*tod*$^-$, white), and TGY medium control (grey).

(*tod*$^+$) and MD417 (*tod*$^-$) containing either toluene, chlorobenzene, or 3,4-dichloro-1-butene. Products were identified from culture supernatants of strain MD560 as toluene *cis*-dihydrodiol, chlorobenzene *cis*-dihydrodiol, and 1,2-dihydroxy-3,4-dichlorobutane, which were derived from toluene, chlorobenzene, and 3,4-dichloro-1-butene, respectively (Table 1). These products were positively identified by comparison with commercially available standards or products isolated from enzyme reactions using purified TDO. *D. radiodurans* strain MD417 failed to oxidize all of the substrates tested.

Expression of TDO in *D. radiodurans*. Incubation of 1.5×10^9 cells/ml of strain MD560 resulted in complete degradation of 25 nmol/ml of either toluene or chlorobenzene in 30 min, and at nearly equal rates; while a similar reaction with 25 nmol/ml of 3,4-dichloro-1-butene resulted in oxidation of about 40% of that substrate in 80 min (data not shown). Incubation of approximately 1 X 10^8 cells/ml *D. radiodurans* strains MD560 and MD417, and *E. coli* (pDTG351)[12], with ^{14}C-labeled toluene resulted in a time-dependent accumulation of ^{14}C-labeled product from *D. radiodurans* MD560 and *E. coli* (pDTG351), both expressing TDO (Fig. 3A). A faster product formation by *E. coli* (pDTG351) was observed, and was probably due to the fact that the *tod* genes are constitutively expressed from a multicopy plasmid in *E. coli* and are driven from a different promoter than that used in *D. radiodurans*, thus probably contributing to an increased intracellular level of TDO in *E. coli* versus *D. radiodurans*. *D. radiodurans* MD417 did not accumulate nonvolatile ^{14}C-labeled product, as expected.

A similar reaction containing 25 nmol/ml TCE, another substrate for TDO, resulted in no discernable loss of the TCE as measured by gas chromatography. A more sensitive assay using ^{14}C-TCE and 2×10^9 cells/ml yielded a detectable increase in nonvolatile ^{14}C-labeled material, which was associated with the cells of MD560 only (Fig. 3B). This was consistent with studies in vivo[11] and in vitro[8] in which ^{14}C-TCE oxidation was shown to inactivate TDO and a reactive intermediate covalently attaches to cellular material.

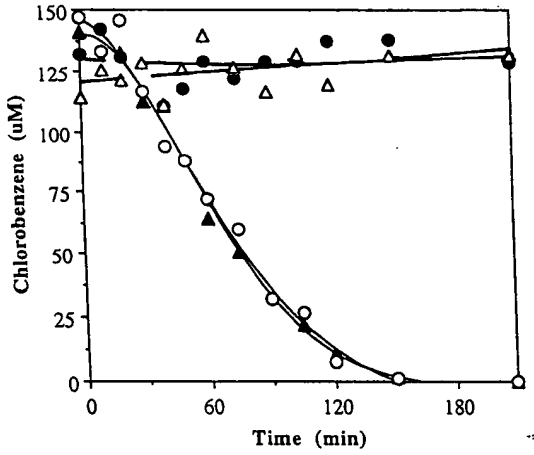

Figure 4. Effect of γ-irradiation (60 Gy/h) on the function of TDO expressed in *D. radiodurans*. Strain MD560 (*tod*$^+$) in the presence of (▲) and absence of (○) irradiation; and MD417 (*tod*$^-$) in the presence of (●) and absence of (△) irradiation.

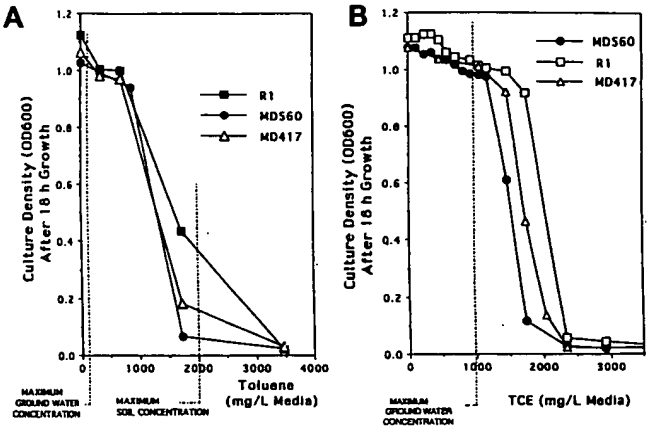

Figure 5. Effect of (A) toluene and (B) TCE on the growth of *D. radiodurans* strains R1, MD417 (vector control, *tod*⁻), and MD560 (*tod*⁺).

Expression of TDO in the presence of radiation. To test the ability of strain MD560 to functionally express TDO under irradiating conditions, strains MD560 and MD417 were first grown in the irradiator (60 Gy/h) for 24 h, to the stationary phase of their growth cycle (OD_{600}=1.1). Cells were then diluted (1:20) in fresh TGY broth and regrown in the irradiator to a cell density of 9×10^7 cells/ml (OD_{600}= 0.9; late exponential growth phase) before being concentrated to 5×10^8 cells/ml in fresh TGY media. Concentrated cells were incubated with 125 nmol/ml chlorobenzene in the presence of high-level radiation. As controls, strains MD560 and MD417 were grown and tested outside the irradiator in a manner identical to that described for irradiated samples. Strain MD560 oxidized 125 nmol/ml of chlorobenzene completely within 2.5 h with similar rates irrespective of the presence or absence of radiation (Fig. 4). Control strain MD417, lacking the *tod* genes, was unable to degrade the chlorobenzene.

Resistance of *D. radiodurans* to toluene and TCE. The effects of solvent concentration on growth of *D. radiodurans* strains R1, MD417, and MD560 were tested. The growth of *D. radiodurans* strains was not substantially affected up to 800 mg/L for toluene and up to 1200 mg/L TCE. These levels are well above those reported at sites containing contaminated groundwaters and many of those containing contaminated soils[3] (Fig. 5).

Discussion

TDO was chosen for expression in *D. radiodurans* because it is prototypic of a large class of bacterial dioxygenases and has a broad substrate range that includes compounds present at sites containing organic and radioactive mixed wastes. Furthermore, TDO comprises three protein components with their attendant metal and organic cofactors[28]; thus, its successful expression in *D. radiodurans* suggests that many less complex biodegradative enzyme systems could be expressed.

Strain MD560, expressing TDO, oxidized indole, toluene, chlorobenzene, and 3,4-dichloro-1-butene—all known substrates for TDO—to the anticipated oxidation products (Table 1). *D. radiodurans* strains grew under continuous irradiating conditions of 60 Gy/h in a ^{137}Cs irradiator (Fig. 1). Furthermore, strain MD560 synthesized functional TDO under those conditions and degraded 125 nmol/ml chlorobenzene while being exposed to radiation (Fig. 4).

The cell envelope of *D. radiodurans* includes an outer and inner lipid membrane that surrounds the cell wall[32,33]. It was not known whether the membrane architecture of this organism might result in sensitivity or resistance to organic solvents. Organic solvents are generally toxic to bacteria as they render their membranes porous[34,35]. Toluene and TCE are two of the most common organopollutants at radioactive DOE waste sites[3]; toluene has been reported at levels as high as 26 mg/L of groundwater and 2000 mg/kg of soil, and TCE at levels as high as 1000 mg/L and 12,000 mg/kg, respectively. *D. radiodurans* strains R1, MD560, and MD417 were all found to be naturally tolerant to toluene and TCE groundwater concentrations well above those found at most sites, and resistant to about half the highest toluene concentrations reported in contaminated soils (Fig. 5).

The lack of TCE oxidation, beyond that measured using a sensitive ^{14}C assay, has been attributed to turnover-dependent TDO inactivation[31]. Enzymatic TCE oxidation is known to generate reactive acyl chlorides that bind covalently to proteins and other macromolecules[8]. Sustained biological TCE oxidation may, thus, require the intracellular biosynthesis of a scavenging nucleophile, such as glutathione, to protect against enzyme inactivation. Our analysis of genome sequences failed to detect DNA homologs to *E. coli* genes *gshA* and *gshB* (data not shown), but it may be possible to clone and express these glutathione biosynthetic genes in *D. radiodurans*.

Experimental protocol

Growth of cells. *D. radiodurans* and *E. coli* strains were grown in TGY medium[18] and Luria-Bertani medium, respectively, with aeration on rotary shakers at 32°C and 37°C, respectively. For assays of TDO activity in the presence or absence of radiation, the *D. radiodurans* strains, and *E. coli* strains (pDTG351 [ref. 12] and pDTG601a [ref. 12] expressing TDO) were grown at ambient room temperature (about 24°C). Kanamycin was used at a concentration of 8 μg/ml for recombinant *D. radiodurans* strains and 30 μg/ml for *E. coli* (pDTG351). For *E. coli* (pDTG601a) ampicillin was used at a concentration of 50 μg/ml. Typically, cell densities were determined by OD_{600} measurements, where OD_{600} 1.0 was equal to 1×10^8 cells/ml for *D. radiodurans*. *D. radiodurans* strains typically do not exceed 1.2×10^8 cells/ml when grown in TGY medium.

Strain construction. The tandem duplication vector pMD417 and *D. radiodurans* control strain MD417 (lacking *tod* genes) have been described[21]. Strain MD560 is identical to strain MD417 except for the presence of the *todC1C2BA* genes. An EcoRI-BamHI (4.2 kb) fragment containing the *todC1C2BA* genes[12] was cloned from plasmid pHG2 (ref. 11) into pMD417 forming plasmid pMD532. MD560 is the product of transformation of wild-type strain R1 with pMD532 followed by selection on TGY plates containing kanamycin. pMD532 cannot replicate as a plasmid in *D. radiodurans* because of the absence of a deinococcal plasmid origin of replication. Upon transformation, integration of pMD532 into the chromosomal target sequence occurs by homologous recombination (a single crossover between the *BC* regions of the plasmid and the chromosome, respectively). As a result, the integrated vector becomes flanked on both sides by chromosomal *BC* sequences, forming a chromosomal tandem duplication. In *D. radiodurans*, chromosomal region 560 can confer resistance to kanamycin (encoded by a portion of the *E. coli* plasmid pMK20 that contains the *aphA* gene). Transcription of the *aphA* gene is driven by deinococcal constitutive promoting sequences in a fragment derived from the *D. radiodurans* SARK natural plasmid pUE11 (ref. 18). Transcription of the TDO genes in strain MD560 are driven by deinococcal constitutive promoting sequences in a fragment derived from the *D. radiodurans* SARK natural plasmid pUE10[18].

Product isolation and analysis. Indigo production from indole was detected from *D. radiodurans* strain MD560 by growing it to mid-log phase (OD_{600} 0.5) in 100 ml of TGY, and then incubating the culture overnight with 100 mg of indole. Indigo was also detected in isopropyl-β-D-thiogalactopyranoside–induced *E. coli* (pDTG601a). Indole was added in solid form to the cultures. Following overnight incubation, cells were removed and the supernatants extracted twice with an equal volume of ethyl acetate. The ethyl acetate was evaporated under vacuum to a final volume of 5 ml, and 50 μl spotted onto a 5×20 cm silica thin layer chromatography (TLC) plate. Separation by TLC was carried out using ethyl acetate as the mobile phase. Commercial indigo, as well as indigo produced from *E. coli* (pDTG601a) incubations with indole, served as comigrating controls. The ratio of indigo migration to the solvent front (R_f) was determined to be 0.84. For determination of the products of toluene, chlorobenzene, and 3,4-dichlorobutane degradation by strain MD560, each was added individually as 100 μl to 100 ml cultures of mid-log phase MD560 and MD417 (OD_{600} 0.5) and allowed to incubate overnight for 12–16 h. Following overnight incubation, the toluene and chlorobenzene *cis*-dihydrodiols, and 1,2-dihydroxy-3,4-dichlorobutane, respectively, were extracted from culture supernatants with two volumes of ethyl acetate and analyzed by gas chromatography/mass spectrometry (GC/MS) on a Hewlett Packard (Paramus, NJ) 6890 gas chromatograph equipped with an HP5973 mass selective detector, 30 m HP5-MS column,

split/splitless inlet at 220°C, and an oven temperature profile of: 50°C for 3 min, 50–300°C at 10°C/min, and then 300°C for 2 min. All data were collected and analyzed using a Hewlett Packard Chemstation. All data were consistent with data for products isolated from reactions of the same substrates with purified toluene dioxygenase, and with previously reported values for those products[36,37]. The 3,4-dichlorobutane-1,2-diol product was analyzed on a Kratos (Ramsey, NJ) GC/MS-25 mass spectrometer with direct port inlet and chemical ionization using isobutane as carrier gas.

Analysis of substrate degradation. Toluene, chlorobenzene, 3,4-dichloro-1-butene, and TCE degradation studies were measured initially in 11 ml sealed reaction vials with 1 ml of concentrated cells at 1.5×10^9 cells/ml and 25 nmol/ml of substrate. At time points over a 1 h period, 50 ml of headspace was removed from reaction mixtures with a gas-tight syringe and analyzed on a Hewlett Packard 5890 GC equipped with flame ionization detector and a DB-1 capillary column (0.25 mm in diameter, 0.25 mM film thickness, 30 m length) and operating at an isothermal oven temperature of 180°C, splitless injection at a temperature of 250°C, with peak integration. ^{14}C-TCE experiments were conducted in sealed 11 ml vials using strains MD560, MD417, and a TGY negative control, to which 20 µl of ^{14}C-TCE (8.5 mM in dimethylformamide (DMF), specific activity 5.4 µCi/µmol), was added to 1 ml of cells concentrated to a density of 2×10^9 cells/ml. A zero time point and an 18 h time point were taken by removing 20 µl of the incubation mixture and applying the 20 µl to a 1×1 cm silica TLC plate. Once dry, the TLC plates were added to 5 ml of scintillation cocktail and the residual nonvolatile ^{14}C was measured using a scintillation counter. The majority of radioactivity was associated with cellular material as determined by separation of cells from supernatants by centrifugation and analysis of each by a scintillation counter. The background levels of radioactive material associated with each sample were determined to be due to contaminating traces of ^{14}C-dichloroacetic acid by high pressure liquid chromatography analysis, and was associated with the supernatants of incubation mixtures. Similar methodology was used for conducting the ^{14}C-toluene assays as was used for ^{14}C-TCE experiments, except that 25 µl ^{14}C-toluene (specific activity 56.24 µCi/µmole; 377.8 µM in DMF) were added to 1 ml of cells adjusted to OD_{600} 1.0 for both *D. radiodurans* and *E. coli* strains. The final concentration of ^{14}C-labeled toluene was 9.5 µM, and the incubations were monitored by removing 20 µl at timed points over a 6 h period.

Growth of *D. radiodurans* and expression of TDO in the presence of radiation. Strains MD560 and MD417 were grown in the presence of continuous γ-irradiation (60 Gy/h) in a ^{137}Cs Gammacell 40 irradiation unit (Atomic Energy of Canada Limited [Ottawa]) at 24°C. *E. coli* (wild-type strain AB1157) was used as a negative growth control. Survival rates were determined by plating appropriate dilutions of irradiated cells and counting the colony forming units on plates. For chlorobenzene degradation studies, strains MD560 and MD417 were first grown inside the irradiation unit for 24 h to an OD_{600} of 1.1 (1.1×10^8 cells/ml). These cells were diluted 1:20 with fresh TGY and regrown in the presence of radiation to OD_{600} 0.9. The cells were then removed temporarily from the irradiation unit and concentrated to 5×10^8 cells/ml. Unirradiated control cells (MD417 and MD560) were similarly grown and concentrated. 0.5 ml of concentrated cells was aliquoted to each of thirteen 11 ml sealed vials and then 125 nmol of chlorobenzene were added to each from a 5 mM stock chlorobenzene solution prepared in dH_2O the day before. Following the addition of substrate, the vials containing irradiator-grown cells were immediately placed back into the irradiator for incubation. At prespecified time points, 0.5 ml of ethyl acetate was injected into the 11 ml vials, and the sample was vigorously shaken and then frozen at -20°C. For quantitative analysis, the ethyl acetate fraction was collected, water was removed by treatment with anhydrous sodium sulfate, and then 2 µl were analyzed by quantitative GC/MS analysis using an HP6890 GC with HP5973 mass selective detector as described above except that selective ion monitoring was used for chlorobenzene molecular ion at *m/z* 112. Standard curves were prepared in similar fashion using TGY medium and varying the amount of chlorobenzene added.

Resistance of *D. radiodurans* to toluene and TCE. *D. radiodurans* strains R1, MD560, and MD417 were grown overnight in liquid growth medium and then subcultured in duplicate to an OD_{600} of 0.02 in fresh medium with varying amounts of toluene or TCE added to each. After 18 h incubation, the cell densities were determined and plotted as a function of solvent concentration.

DNA manipulation. DNA cloning, preparation, and transformations were as described previously[18,20,21,38].

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Engineering *Deinococcus geothermalis* for Bioremediation of High-Temperature Radioactive Waste Environments

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***Deinococcus geothermalis* is an extremely radiation-resistant thermophilic bacterium closely related to the mesophile *Deinococcus radiodurans*, which is being engineered for in situ bioremediation of radioactive wastes. We report that *D. geothermalis* is transformable with plasmids designed for *D. radiodurans* and have generated a Hg(II)-resistant *D. geothermalis* strain capable of reducing Hg(II) at elevated temperatures and in the presence of 50 Gy/h. Additionally, *D. geothermalis* is capable of reducing Fe(III)-nitrilotriacetic acid, U(VI), and Cr(VI). These characteristics support the prospective development of this thermophilic radiophile for bioremediation of radioactive mixed waste environments with temperatures as high as 55°C.**

The bacterium *Deinococcus geothermalis* (13) is remarkable not only for its extreme resistance to ionizing radiation but also for its ability to grow at temperatures as high as 55°C (13) and in the presence of chronic irradiation (8). The organism was isolated by Ferreira et al. (13) from hot springs together with *Deinococcus murrayi*. Both bacteria are moderately thermophilic and belong to the bacterial family *Deinococcaceae* (4, 7, 22), currently comprised of seven distinct nonpathogenic radiation-resistant species, of which *Deinococcus radiodurans* strain R1 is the best characterized (4). Advances in genetic engineering for *D. radiodurans* (9–12, 29) were a stimulus for its genome sequencing (17, 33), annotation (22), and proteomic (18) and transcriptome (19) analyses. The other deinococcal species have been reported as nontransformable or have not yet been tested for transformability by chromosomal or plasmid DNA and have been left unexplored by recombinant DNA technologies. Other genetic approaches including conjugation and protoplast fusion have not been successful in the *Deinococcaceae* (16).

A present genetic engineering goal for *D. radiodurans* is its development for bioremediation of U.S. Department of Energy (DOE) mixed radioactive environmental waste sites left over from nuclear weapons production during the Cold War (21, 25, 27, 28). These sites contain immense volumes of waste (3×10^6 m^3) that include radionuclides, heavy metals, and toxic organic compounds and have contaminated 40 million cubic meters of soil and 4 trillion liters of groundwater since 1946 (1, 21, 25, 27, 28). While there has been significant progress in engineering *D. radiodurans* for remediation of radioactive DOE waste environments (5, 8, 15), prospective treatment of contaminated sites with engineered *D. radiodurans* will be limited to temperatures below 39°C, its maximum growth temperature. However, there is a need to develop bioremediating bacteria that are resistant to both radiation and high temperatures because of the existence of thermally insulated contaminated environments where temperatures are elevated by the decay of long-lived radionuclides (e.g., ^{137}Cs and ^{90}Sr) (1). For example, soil columns beneath at least 67 radioactive leaking tanks at DOE's Hanford Site in south-central Washington State have been contaminated and have recorded temperatures as high as 70°C at depths of greater than 18 m (1). Since *D. geothermalis* and *D. murrayi* are both radiation resistant and thermophilic, they have become desirable targets for genetic development of bioremediating strains similar to those developed for *D. radiodurans* (5, 8, 15) but capable of survival and growth at higher temperatures. Given the need to develop bioremediating bacteria for treatment of radioactive high-temperature waste environments, *D. geothermalis* and *D. murrayi* were tested for their transformability with the autonomously replicating *Escherichia coli-D. radiodurans* shuttle plasmid pMD66 (9), which expresses kanamycin (KAN) and tetracycline (TET) resistance in *D. radiodurans* and additionally expresses ampicillin resistance in *E. coli*.

pMD66 and its numerous derivatives (9–12) have been used successfully to functionally express cloned genes in *D. radiodurans* growing under chronic irradiation. Examples include the *mer* operon of *E. coli* (5), which encodes Hg(II) resistance and reduction, and the *Pseudomonas* operon *todC1C2BA* (15), which encodes partial degradation of toluene. The present work shows that *D. geothermalis* is capable of expressing Hg(II)-reducing functions cloned in pMD66 at elevated temperatures and under chronic radiation and, like *D. radiodurans* (14), is naturally capable of reducing a variety of other metal contaminants present in DOE waste sites.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and transformation. *D. radiodurans* R1 (ATCC BAA-816) (33), *D. geothermalis* DSM11300, and *D. murrayi* DSM11303 were grown in TGY broth (1% Bacto Tryptone, 0.1% glucose, 0.5% Bacto Yeast Extract) (Difco) or minimal medium (MM) (see Table 2) (32). Liquid cultures were inoculated at ~5×10^6 cells/ml. For solid medium, Bacto Agar (Difco) or Noble agar (Difco) was added to TGY or MM, respectively, to 1.5% (wt/vol). *D. radiodurans* was grown at 32°C, and *D. geothermalis* and *D. murrayi* were grown at 37°C or at higher temperatures as indicated. *E. coli* was grown in Luria-Bertani

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TABLE 1. Transformation of pMD66/68 into *D. geothermalis*, *D. radiodurans* and *E. coli*

	No. of transformants/μg of DNA for recipient[a]:		
	D. geothermalis	*D. radiodurans*	*E. coli*
Plasmid source[b]			
pMD66 (purified from *E. coli*)	$1 \times 10^1 \pm 2 \times 10^1$	$1 \times 10^2 \pm 8 \times 10^1$	$9 \times 10^4 \pm 3 \times 10^3$
pMD68 (purified from *D. radiodurans*)	$5 \times 10^1 \pm 2 \times 10^1$	$8 \times 10^5 \pm 1.5 \times 10^4$	$4 \times 10^5 \pm 2.1 \times 10^4$
pMD66 (purified from *D. geothermalis*)	$5 \times 10^2 \pm 29 \times 10^1$	$4 \times 10^5 \pm 7 \times 10^3$	$4 \times 10^2 \pm 25 \times 10^1$

[a] Km^r transformants per microgram of plasmid purified from the indicated strains.
[b] pMD66, pMD68, and pMD66-*D. geothermalis* have identical restriction maps (Fig. 1A).

that irradiation-induced mutations and deletions are rare in *D. geothermalis*, as is the case in *D. radiodurans* (9, 11). These results show that pMD66 is retained in *D. geothermalis* without alteration following high-dose irradiation and recovery and is repaired with similar efficiency to its chromosomes.

Construction and characterization of Hg(II)-resistant *D. geothermalis*. The complete *E. coli* Hg(II) resistance (*mer*) operon (4.2 kb, encoding six proteins) (3, 5) has previously been functionally expressed in *D. radiodurans* by using a pMD66 derivative, pMD727 (5) (Fig. 2A). In *D. radiodurans*, all six *mer* genes are necessary for reduction of Hg(II) to Hg(0). pMD727 was successfully transformed into *D. geothermalis* (Fig. 2B), yielding strain MD865. This construction placed the *mer* genes under the control of a constitutive *D. radiodurans* promoter (P2, Fig. 2A), and Southern analysis with a radiolabeled probe containing a 1.5-kb *Eco*RI-*Bgl*II fragment from the *mer* operon showed no significant homology with the *D. geothermalis* genome (Fig. 2B). Reduction of Hg(II) to volatile elemental Hg(0) by *D. geothermalis* strain MD865 was examined by testing for mercury volatilization, which causes film darkening (5, 26). Following 14 h of incubation with Hg(II) in a microplate at 32 or 40°C, covered by X-ray film, wild-type *D. geothermalis* showed modest Hg(0) volatilization. However, strain MD865 (*D. geothermalis*/*mer*$^+$) showed substantial Hg(0) volatilization based on film darkening compared to wild-type *D. geothermalis* at 32 or 40°C (Fig. 2C). MD865 also was resistant to 50 μM Hg(II) during growth at 50°C (Fig. 2D) and displayed luxuriant growth at 50°C in the presence of 50 Gy/h on solid medium containing 30 μM merbromin (data not shown). Wild-type *D. geothermalis* did not grow in medium containing 30 μM merbromin in the presence or absence of chronic radiation.

Reduction of metals. *D. geothermalis* reduced Fe(III)-NTA in the presence of lactate at 30°C (data not shown) and in the presence of lactate or pyruvate at 45°C (Fig. 3A). At 40°C *D. geothermalis* rapidly reduced Cr(VI) in TGY cultures under both aerobic and anaerobic conditions (Fig. 3B). AQDS (anthraquinone-2,6-disulfonate) is a quinone-containing organic compound that can be utilized as an electron acceptor for respiration and growth by a variety of dissimilatory metal-reducing bacteria (20). As an electron acceptor, AQDS is reduced to the corresponding dihydroquinone (AH_2DS) (20). Reduction of U(VI) by *D. geothermalis* at 40°C occurred only in the presence of AQDS (Fig. 3C). These results are very similar to the reduction capabilities reported for *D. radiodurans* at lower temperatures (14).

Growth characteristics of *D. geothermalis*. *D. geothermalis* was tested for its amino acid utilization and growth on various Embden-Meyerhof-Parnas substrates. Table 2 shows that, in the absence of irradiation, growth of *D. geothermalis* is independent of any amino acids and the bacterium can utilize ammonium sulfate and grow on tricarboxylic acid cycle intermediates. In the presence of chronic irradiation, growth of *D. geothermalis* is less dependent than that of *D. radiodurans* on Cys and Met, or other exogenously provided amino acids (data not shown). Therefore, the metabolism of *D. geothermalis* appears substantially more robust than that in *D. radiodurans*.

DISCUSSION

D. geothermalis is transformable with autonomous plasmids originally constructed for *D. radiodurans*. Thus, experimental advances in the genetic management of *D. radiodurans* over the last decade (5, 8, 24) could facilitate rapid development of *D. geothermalis* for fundamental and practical objectives. *D. geothermalis* is a thermophile (13) with substrate utilization-growth characteristics that are distinct from those of *D. radiodurans* (Table 2). Under nonirradiating conditions, *D. geothermalis* is not dependent on exogenous amino acids for growth and can utilize ammonium sulfate. These characteristics endow the species with the ability to grow in nutritionally restricted environments that do not support the growth of *D. radiodurans* (32). *D. geothermalis* is also able to grow over a broad temperature range extending to 55°C (13) and displays superior growth in the presence of chronic irradiation (50 Gy/h) in nutritionally restricted medium, compared to *D. radiodurans*. While these characteristics support the idea that *D. geothermalis* may be a more robust candidate than *D. radiodurans* for treatment of radioactive waste environments (32), until now

FIG. 2. Construction and characterization of Hg(II)-resistant-reducing *D. geothermalis*. (A) pMD727 (5) was transformed into *D. geothermalis*, giving strain MD865. (B) Southern blot hybridization of *Eco*RI-digested total DNA from *D. geothermalis* (wild type, *mer* negative) and MD865 (*D. geothermalis*/*mer*$^+$) with a radiolabeled *mer* probe. pMD727 contains a unique *Eco*RI (E) site. Molecular size standards: λ/*Hin*dIII, as in Fig. 1A and B. Wild-type strain abbreviations are as in Fig. 1. (C) Hg(0) volatilization assays at 32 and 40°C for *D. geothermalis*, *D. radiodurans*, MD865 and MD735 (*D. radiodurans*/*mer*$^+$), and TGY (growth medium, no cells). (D) Growth curves for MD865 and MD735 in TGY plus 50 μM merbromin [Hg(II)] at 50°C.

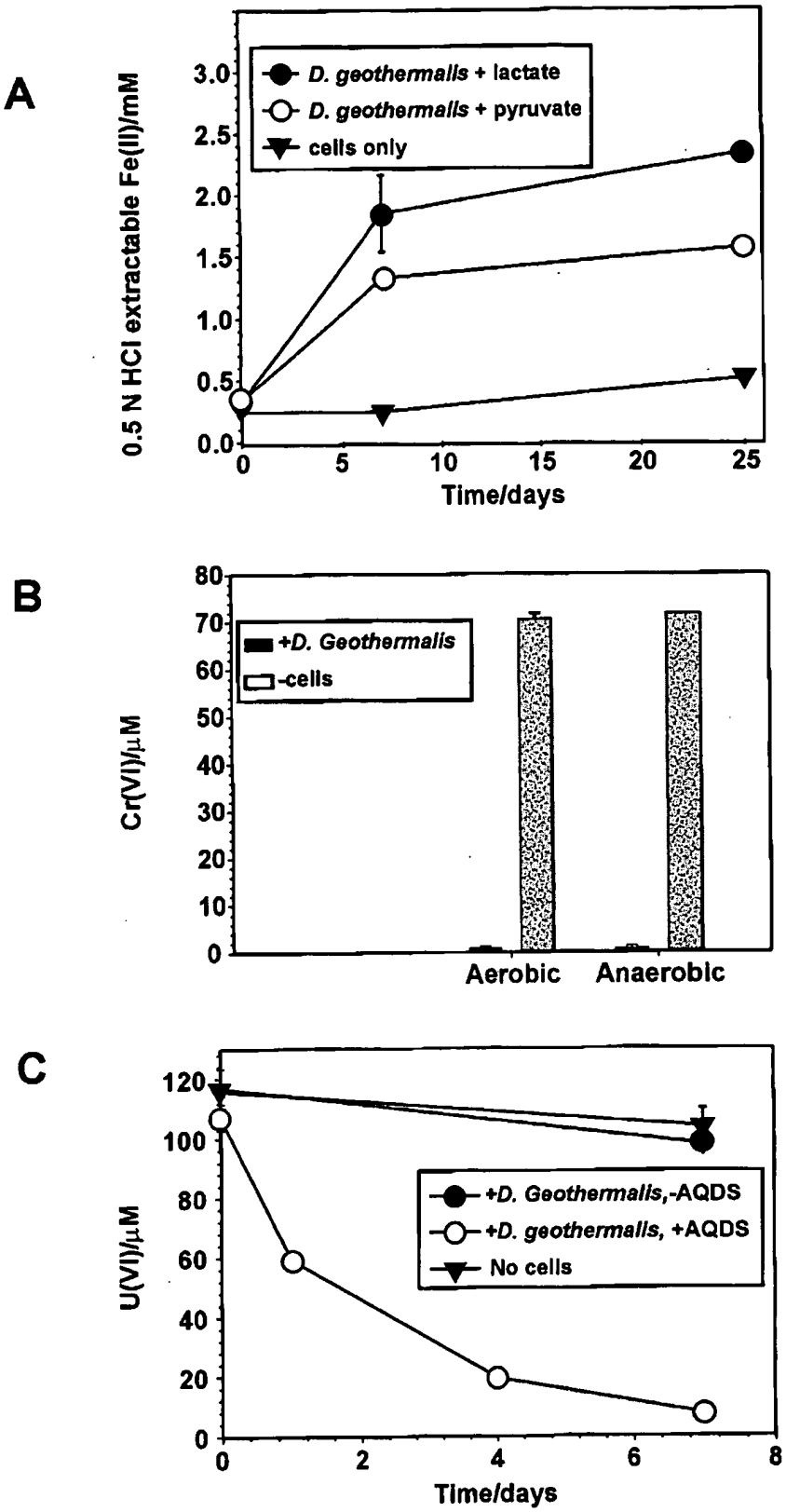

FIG. 3. Metalloreduction by wild-type *D. geothermalis*. (A) Fe(III)-NTA reduction coupled to oxidation of organic substrates at 45°C in the absence of oxygen. (B) Cr(VI) reduction in aerobic or anaerobic conditions as measured by loss of Cr(VI) from solution at 40°C. (C) Reduction of U(VI) in the presence or absence of AQDS as measured by loss of U(VI) at 40°C.

TABLE 2. Growth characteristics of *D. radiodurans* and *D. geothermalis* grown in mm[a]

Substrate[c]	Growth of strain[b]	
	D. radiodurans	*D. geothermalis*
Fructose + C,H,L,A,M,P	+++	+++
Fructose + Met	+++	+++
Fructose −aa +$(NH_4)_2SO_4$	−	+++
Fructose −NAD +Met	−	+++
α-Ketoglutarate	−	++
Succinate	−	++
Fumarate	−	++
Oxaloacetate	+	++
Malate	−	+

[a] Deinococcal cells were grown in deinococcal MM (32) at 32°C. Deinococcal MM contained the indicated Embden-Meyerhof-Parnas substrate (2 mg/ml), NAD (1 μg/ml), methionine (Met) at 50 μg/ml, phosphate buffer (20 mM, pH 7.5), $CaCl_2$ (0.18 mM), and $MgSO_4$ (0.8 mM), and Mn^{2+} (5.4 μM $MnCl_2$) was added as the only transition metal cation.

[b] Growth on substrate: +++, good; ++, moderate; +, poor; −, absent.

[c] Abbreviations: Fructose + C,H,L,A,M,P, fructose plus Cys, His, Lys, Asp, Met, and Pro, each at 50 μg/ml. +Met, only methionine added at 50 μg/ml. −aa, no amino acids added. +$(NH_4)_2SO_4$, ammonium sulfate added to a final concentration of 15 mM. −NAD, no NAD added.

there has been no genetic system available to exploit this species.

Our data show that plasmid-based transformation systems developed for *D. radiodurans* (Fig. 1 and 2) can be used to functionally express cloned genes in *D. geothermalis* at temperatures as high as 50°C (Fig. 2) and in the presence of chronic irradiation. Plasmids introduced into *D. geothermalis* are also efficiently expressed following exposure to high-level acute irradiation (Fig. 1C), without any apparent plasmid loss or mutagenesis. The differential hybridization results with a chromosome- and a plasmid-derived probe in MD865 (*D. geothermalis*/pMD66) (Fig. 1B) support the idea that pMD66 exists in multiple copies in *D. geothermalis*. The survival of *D. geothermalis*/pMD66 plated on TGY-KAN was indistinguishable from that found for wild-type *D. geothermalis* on TGY. As in *D. radiodurans*, this suggests that multiple identical plasmid copies serve as a substrate for efficient repair by homologous recombination (10). Therefore, these studies establish *D. geothermalis* and *D. radiodurans* as the only two extremely radiation-resistant vegetative bacteria that are currently amenable to genetic engineering.

The presence of pMD66 in *D. geothermalis* as a covalently closed circle was confirmed by plasmid rescue in *E. coli* (Table 1) (12), and restriction enzyme mapping and Southern analysis confirmed its predicted structure and stability in *D. geothermalis* (Fig. 1A). When total DNA containing pMD66 was purified from *D. geothermalis* and transformed back into wild-type *D. geothermalis*, there was only a small increase in the number of transformants over that with pMD66 purified from *E. coli*. In contrast, there was a large increase in transformation frequency observed in *D. radiodurans* with pMD66 purified from *D. radiodurans* or *D. geothermalis* over that with pMD66 purified from *E. coli* (Table 1). Therefore, the plasmid transformation capabilities of *D. geothermalis* appear to be significantly less than those of for *D. radiodurans*. While the reasons for this difference are unclear, the fact that pMD66 purified from *D. geothermalis* could be used to transform *D. radiodurans* at high efficiency, but not *D. geothermalis*, suggests that transport of DNA into *D. geothermalis* is inefficient. Wild-type *D. murrayi* is naturally resistant to KAN and, therefore, was not tested for transformability with pMD66/68. However, *D. murrayi* is sensitive to chloramphenicol and could be a suitable host for plasmids encoding Cm^r, but we found it to be nontransformable with high concentrations of pMD300/308 (10) purified from *E. coli* or *D. radiodurans* and did not investigate this species further.

To demonstrate the utility of *D. geothermalis* for bioremediation purposes, we introduced the highly characterized Hg(II) resistance operon (*mer*) of *E. coli* (3) into *D. geothermalis* on an autonomously replicating *D. radiodurans* plasmid (Fig. 2A). Ionic Hg(II) is a prevalent contaminant of radioactive DOE waste sites, where the highest concentration level in contaminated areas has been reported as 10 μM (28). When present in *D. radiodurans*, the *mer* operon confers Hg(II) resistance and endows cells with the ability to reduce highly toxic Hg(II) to much less toxic elemental Hg(0) (5). Similarly, we show that strain MD865 (*D. geothermalis*/*mer*$^+$) is (i) resistant to the bactericidal effects of ionic Hg(II) at concentrations (50 μM; Fig. 2D) well above the highest concentration reported for Hg(II)-contaminated DOE waste sites, (ii) able to reduce toxic Hg(II) to much less toxic elemental and volatile Hg(0) (Fig. 2C), and (iii) able to functionally express the *mer* operon in highly irradiating environments (50 Gy/h) at temperatures as high as 50°C. It is notable that the mesophilic *E. coli* Mer proteins (3) were functional in *D. geothermalis* growing at 50°C. While mechanisms underlying thermophilicity appear to be complex and currently are not well characterized (23), there is some precedent for the interchangeability of genes from mesophiles and thermophiles. For example, the aspartate aminotransferase gene (*aspATSs*) of the hyperthermophile *Sulfolobus solfataricus* has been functionally expressed at mesophilic temperatures in *E. coli* (2). We believe that numerous other metal resistance functions from other bacteria, specific for other metals, could be cloned into *D. geothermalis* by this approach.

It was recently shown that under strict anaerobic conditions *D. radiodurans* can reduce Fe(III)-NTA coupled to the oxidation of lactate to CO_2 and acetate (14). *D. radiodurans* could also reduce U(VI) or Tc(VII) in the presence of AQDS and could directly reduce Cr(VI) in both anaerobic and aerobic conditions (14). The enzymatic reduction of multivalent metals and radionuclides can have a major impact on their solubility and, hence, mobility in the environment. Such changes in solubility make microbial metal reduction a suitable process for immobilizing metals and radionuclides within contaminated environments in situ (8, 25). Localized contaminated sediments and soils at DOE sites can have temperature levels that exceed those that can be tolerated by *D. radiodurans*. We show that the *D. geothermalis* suite of metal-reducing capabilities appears to be very similar to that reported in detail for *D. radiodurans* (14) but functional at higher temperatures (Fig. 3).

We are not aware of expression of any cloned genes in *D. geothermalis* previous to this report. Our demonstration that plasmids developed for *D. radiodurans* are functional in *D. geothermalis* strongly supports the idea that bioremediating gene constructs developed for *D. radiodurans* could be transferred to *D. geothermalis*. This could yield metabolically proficient, extremely radiation-resistant, and thermophilic bacteria suit-

able for the treatment of high-temperature mixed radioactive wastes.

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